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ORIGINAL ARTICLE

Antibacterial activity of traditional spices against lower respiratory tract pathogens: combinatorial effects of *Trachyspermum ammi* essential oil with conventional antibiotics

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Significance and impact of the study: Multidrug-resistant bacteria associated with infections of the lower respiratory tract have become a significant public health concern. Therefore, known antimicrobial agents derived from plants (e.g. essential oils) and their synergistic combinations with conventional antibiotics may overcome the antimicrobial resistance. In this respect, the antibacterial effects of ajowan essential oil, thymol and their combinations with antibiotics were assessed against some standard strains and drug-resistant clinical isolates of major respiratory bacteria (*Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pneumoniae*).

Keywords
antibiotics, antimicrobials, resistance, staphylococci, streptococci.

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Abstract

The aim of this study was to investigate the effects of ajowan essential oil (AjEO)/thymol and antibiotics combinations against three standard strains and six resistant clinical isolates of major respiratory bacteria (*Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae*). The broth microdilution method was conducted to determine the minimum inhibitory concentrations (MIC) of essential oil/thymol and antibiotics. The checkerboard method was used to investigate the interactions between the essential oil/thymol and antibiotics by means of the fractional inhibitory concentration index (FICI). The chemical composition of essential oil was also analysed by GC–MS and GC-FID. Thymol (50·75%), γ-terpinene (25·94%) and *p*-cymene (18·31%) were identified as major constituents of the oil. The most sensitive organisms to ajowan volatile oil were *Strep. pneumoniae* bacteria (MIC = 0·125–0·5 mg ml^{-1}). Synergistic effects were observed with AjEO/thymol and amoxicillin combinations on methicillin-resistant *Staph. aureus* clinical isolates (FICI = 0·37–0·50) and with essential oil and ciprofloxacin combinations against *P. aeruginosa* ATCC 27853, *Staph. aureus* ATCC 25923 and penicillin (P)-resistant *Strep. pneumoniae* bacteria (FICI = 0·37–0·50). Combination of thymol and ciprofloxacin produces synergistic effects only against *P. aeruginosa* ATCC 27853 and P-resistant *Strep. pneumoniae* clinical isolate (FICI = 0·46–0·49).

Introduction

Drug- and multidrug-resistant (MDR) bacteria are a significant cause of both health care- and community-associated infections including those of the lower respiratory tract. *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* bacteria are among the ones most commonly associated with antibiotic resistance and they can even serve as markers for this phenomenon. Methicillin (Met)-resistant *Staph. aureus* (MRSA) bacteria

are frequently involved in the development of nosocomial pneumonia and nosocomial bloodstream infections (David and Daum 2010). The resistant strains of *Strep. pneumoniae* are the major pathogens leading to community acquired infections such as pneumonia, meningitis or acute otitis media (Ziglam and Finch 2002). *P. aeruginosa*, a metabolically versatile pathogen, causes a broad spectrum of severe opportunistic infections, being a major factor of morbidity and mortality (Porras-Gómez *et al.* 2012). According to the European Centre for Disease Prevention and Control (ECDC), the prevalence of nosocomial infections caused by MRSA strains exceeds 25% in eight out of the 28 member states of the European Union (EU), including Romania (ECDC 2010). Every year, about 25 000 patients die in the EU due to an infection caused by MDR-bacteria (Freire-Moran *et al.* 2011). Besides, in the EU, extra health care costs as well as productivity losses due to infections with MDR-bacteria amounts to about 1·5 billion euros annually (European Commission 2011). The prevention and therapeutic control of MDR-bacteria is a priority among public health issues in the EU (Köck *et al.* 2010).

To ensure therapeutic control of MDR-bacteria it is imposed to find new antimicrobial agents or to increase the antibiotics' activity by overcoming the resistance. Plants are a rich reservoir of antimicrobial agents and essential oils are among the most promising vegetal metabolites. The combinations between known antibiotics and essential oils represent an interesting concept to overcome the antimicrobial resistance (Fadli *et al.* 2014; Kasrati *et al.* 2014; Aelenei *et al.* 2016). Essential oils play an important role in the protection of plants acting as antibacterial, antifungal, antiviral and insecticide agents; they have also a long history of use as antimicrobial agents in the pharmaceutical, sanitary, cosmetic, agricultural and food industries (Burt 2004). Essential oils may restore sensitivity to antibiotics, decrease the effective dose of antibiotics thus minimizing their side effects, extend the antimicrobial spectrum and reduce the cost of anti-infective therapy (Fadli *et al.* 2012).

Ajowan, *Trachyspermum ammi* (L.) Sprague ex Turrill (Apiaceae) is an annual herbaceous and aromatic plant, originating in India and Egypt. It has a long history of use as a cooking spice and herbal remedy, especially in the Asian regions (Singh and Choudhary 2008). The fruits of ajowan's are the most used part. They have a bitter, pungent taste and they develop trough crushing a strong aromatic fragrance, resembling that of thyme. Their characteristic aroma is due to the essential oil (2·5–5%) containing thymol as its major constituent (35–60%), followed by *p*-cymene, γ-terpinene and β-pinene (Kaur and Arora Singh 2010; Bairwa *et al.* 2012). Ajowan dried fruits, whole or powdered, are used for flavouring foods, snacks, sauces and vegetable preparations or as preservative in the food industry or in alcoholic beverages (Malhotra and Vijay 2004). Besides their extensive use as a spice, the fruits of ajowan have also been used in traditional Indian herbal medicine as a remedy for a wide array of unrelated ailments including indigestion and colic, dyspepsia, flatulence, diarrhoea, arthritis and rheumatism, coughs, nasal congestion, pharyngitis, bronchial pneumonia or asthma (Zachariah 2008; Vitali *et al.* 2016). Apart from the traditional use, in the last decade, several studies have reported a marked antimicrobial activity of ajowan essential oil (AjEO) against a broad spectrum of bacterial strains, both standard strains and clinical isolates with different levels of antibiotic resistance, such as: *Staph. aureus,* streptococci including *Strep. pyogenes, Klebsiella pneumoniae, Acinetobacter baumanii, Escherichia coli,* enterococi*, Vibrio cholerae*, with minimum inhibitory concentrations (MIC) <2% (v/v) (Mayaud *et al.* 2008; Mahboubi and Kazempour 2011). The AjEO proved to be more active than other essential oils recognized as a powerful antimicrobial such as thyme, clove or tea tree oils (Mayaud *et al.* 2008).

Also, the antimicrobial activity of thymol, the main phenol of AjEO, has been extensively studied and reported in literature (Dorman and Dean 2000; Nostro *et al.* 2004; Benelli *et al.* 2017; Tabari *et al.* 2017; Ngahang Kamte *et al.* 2018; Pavela *et al.* 2018). *Staph. aureus, Proteus mirabilis, Proteus vulgaris, Escherichia coli, Staph. epidermidis* and *Bacilllus subtilis* bacteria are some of the most sensitive strains to thymol (Marchese *et al.* 2016). Some studies have also identified a good antimicrobial activity of thymol against *P. aeruginosa* (Marchese *et al.* 2016). Moreover, thymol and thymol-containing plants are efficient in the prevention and treatment of respiratory tract infections.

In this respect, AjEO is a plausible candidate for the study on its interactions with antibiotics and the effects of such combinations on antimicrobial resistance. The present study aimed to investigate the effects of AjEO, alone and in combination with antibiotics (amoxicillin (Amx), ciprofloxacin), against standard strains and antibiotic resistant clinical isolates of major respiratory pathogens (*Staph. aureus*, *Strep. pneumoniae* and *P. aeruginosa*). In addition, the effects of thymol in combination with Amx and ciprofloxacin were also evaluated for comparative purposes. To the best of our knowledge, the effects of AjEO, thymol and their combinations with antibiotics against clinical isolates of respiratory tract pathogens have not been investigated so far.

Results and discussion

Chemical composition of AjEO

GC and GC–MS analysis revealed the presence of 16 components, which accounted for 98·93% of the total

essential oil. The major constituent was thymol (50·75%), followed by γ-terpinene (25·94%), *p*-cymene (18·31%) and β-pinene (2·27%) (Table 1). Our result is in agreement with previous literature data reporting high levels of thymol (33·98–80·70%) in the essential oil of ajowan fruits (Mayaud *et al.* 2008; Kumar *et al.* 2011; Mahboubi and Kazempour 2011; Souren *et al.* 2011).

Antimicrobial susceptibility assessment

The clinical isolates showed a characteristic antimicrobial resistance profile. Mainly the *Staph. aureus* and *P. aeruginosa* isolates were MDR strains, exhibiting resistance to two or three antibiotics from different classes (Table 2). Taking into account the common resistance characteristics of the isolates from each bacterial species and to simplify the exposure, we generally appreciated these pathogens as follows: Amx-resistant *P. aeruginosa*, Met-resistant *Staph. aureus* and penicillin (P)-resistant *Strep. pneumoniae* bacteria.

Antibacterial activity

Among tested bacteria, both standard and clinical strains of *Strep. pneumoniae* were most sensitive to AjEO (MIC = 0·125–0·50 mg ml^{-1}). Thymol was mostly active against *P. aeruginosa* ATCC 27853 (MIC = 0·07 mg ml^{-1}) and *Staph. aureus* ATCC 25923 (MIC = 0·003 mg ml^{-1}) bacteria (Table 3). Althunibat *et al.* (2016) have found that *P. aeruginosa* MDR are more sensitive to thymol as a pure compound than to essential oil of *Thymus capitatus*. Compared to other constituents of essential oils, thymol has a high capacity to disturb the outer membrane of Gram-negative bacteria such as *P. aeruginosa* (Kon and Rai 2012). It is mainly related to the hydrophobicity of the aromatic nucleus and to the position of hydroxyl group on the ring (Althunibat *et al.* 2016). Also, the higher susceptibility of *Staph. aureus* standard strain and clinical isolates to thymol than to AjEO (0·003 *vs* 4 mg ml^{-1}, and 0·125 *vs* 8 mg ml^{-1}, respectively) might be due to the fact that γ-terpinene and *p*-cymene, constituents of AjEO that usually potentiate the antibacterial effects of thymol (Ultee *et al.* 2002; Souren *et al.* 2011), have a considerable lower toxicity against *Staph. aureus* in comparison with thymol (Cristani *et al.* 2007).

Table 1 The chemical composition of AjEO

Compound	RI*	RI†	% ±SD
Monoterpene hydrocarbons			
α-thujene	922	924	0·41 ± 0·01
α-pinene	928	933	0·21 ± 0·02
Camphene	943	946	0·01 ± 0·00
β-pinene	974	974	2·27 ± 0·04
Myrcene	987	988	0·49 ± 0·03
Δ-3-carene	1004	1008	0·02 ± 0·00
α-terpinene	1013	1014	0·28 ± 0·02
γ-terpinene	1067	1064	25·94 ± 0·61
Terpinolene	1082	1084	0·06 ± 0·01
Monoterpene alcohols			
Terpinen-4-ol	1174	1174	0·10 ± 0·01
Monoterpene ketones			
Piperitenone	1339	1340	0·01 ± 0·00
Aromatic hydrocarbons			
p-cymene	1029	1030	18·31 ± 0·58
Phenols			
Thymol	1296	1295	50·75 ± 0·25
Phenol methyl ethers			
(*E*)-anethole	1280	1282	0·03 ± 0·00
Eugenol	1351	1356	0·03 ± 0·00
Sesquiterpenes			
(*E*)-caryophyllene	1409	1411	0·01 ± 0·00
Total			98·93 ± 1·58

*Retention indices relative to *n*-alkanes (C_8–C_{20}) calculated on a DB-5MS capillary column.
†Retention indices reported in literature.

Table 2 The antimicrobial resistance profile of lower respiratory tract clinical isolates

Clinical isolate	Antimicrobial resistance/inhibition zone of bacterial growth (mm)
P. aeruginosa 2351	Amx/5 (R); Lev/10 (R); Cef/9 (R)
Staph. aureus 37	Met/7 (R); Dox/10 (R); Ery/23 (S); Van/24 (S)
Staph. aureus 4185	Met/6 (R); Dox/10 (R); Ery/11 (R); Van/23 (S)
Strep. pneumoniae 4423	P/6 (R); St/9 (R); Ery/10 (R); Van/21 (S)
Strep. pneumoniae 4546	P/7 (R); St/20 (S); Ery/20 (S); Van/21 (S)
Strep. pneumoniae 4566	P/7 (R); St/22 (S); Ery/23 (S); Van/23 (S)

Amx, amoxicillin; Cef, cefpirome; Dox, doxycycline; Ery, erythromycin; Lev, levofloxacin; Met, methicillin; P, penicillin; St, streptomycin; Van, vancomycin; R, resistance; S, susceptible.

Interactions between AjEO/thymol and antibiotics

Synergistic interactions were found for some AjEO–Amx combinations against *Staph. aureus* ATCC 25923 and MRSA 37 (FICI = 0·37–0·50) (Fig. 1a), resulting an eightfold reduction in MIC of Amx ($0{\cdot}25 \times 10^{-3}$ *vs* 2×10^{-3} mg ml^{-1}) in the case of the best combination (Table 4). Thymol behaves similarly against MRSA 37 (FICI = 0·36–0·49) (Fig. 1b), the best combination leading also to an eightfold reduction in MIC of Amx ($0{\cdot}25 \times 10^{-3}$ *vs* 2×10^{-3} mg ml^{-1}) (Table 4). Also, additive effects were observed for AjEO–Amx and thymol–Amx combinations against *Staph. aureus* ATCC 25923, as well as against MRSA 4185 (Fig. 1a,b) and PRSP 4423 clinical isolates (Table 4).

A synergistic interaction was noted for two AjEO–ciprofloxacin combinations against *Staph. aureus* ATCC 25923

Table 3 Minimum inhibitory concentrations (MICs) of AjEO/thymol and antibiotics (mg ml^{-1})

Standard strains/ Clinical isolates	MIC			
			Antibiotics	
	AjEO	Thymol	Amoxicillin	Ciprofloxacin
Gram-negative bacteria				
P. aeruginosa ATCC 27853	16	0·07	4×10^{-3}	1×10^{-3}
ARPA 2351	16	8	32×10^{-3}	2×10^{-3}
Gram-positive bacteria				
Staph. aureus ATCC 25923	4	0·003	2×10^{-3}	$0{\cdot}5 \times 10^{-3}$
MRSA 37	8	0·125	2×10^{-3}	4×10^{-3}
MRSA 4185	8	0·125	4×10^{-3}	4×10^{-3}
Strep. pneumoniae ATCC 49619	0·25	4	$0{\cdot}07 \times 10^{-3}$	$0{\cdot}5 \times 10^{-3}$
PRSP 4423	0·125	0·125	4×10^{-3}	2×10^{-3}
PRSP 4546	0·50	0·25	$0{\cdot}03 \times 10^{-3}$	$0{\cdot}5 \times 10^{-3}$
PRSP 4566	0·25	0·25	2×10^{-3}	$0{\cdot}5 \times 10^{-3}$

ARPA, amoxicillin-resistant *P. aeruginosa;* MRSA, methicillin-resistant *Staph. aureus;* PRSP, penicillin-resistant *Strep. pneumoniae.*

(FICI = 0·37–0·50) (Fig. 1c) and one AjEO–ciprofloxacin combination against PRSP 4423 (FICI = 0·49), causing a fourfold reduction in MIC of ciprofloxacin ($0{\cdot}125 \times 10^{-3}$ *vs* $0{\cdot}5 \times 10^{-3}$ mg ml^{-1} and $0{\cdot}5 \times 10^{-3}$ *vs* 2×10^{-3} mg ml^{-1}, respectively) (Table 5). Also, one thymol–ciprofloxacin combination produced a synergistic effect against PRSP 4423 (FICI = 0·49); in this case, thymol reduced MIC of ciprofloxacin by fourfold ($0{\cdot}5 \times 10^{-3}$ *vs* 2×10^{-3} mg ml^{-1}) (Table 5). Additive interactions against Met-resistant *Staph. aureus* clinical isolates (MRSA 37, MRSA 4185) and *Strep. pneumoniae* pathogens (*Strep. pneumoniae* ATCC 49619, PRSP 4546, PRSP 4566) were also detected for AjEO–ciprofloxacin combinations (Table 5). Besides, thymol–ciprofloxacin combinations caused additive interactions against *Strep. pneumoniae* and *Staph. aureus* pathogens (Table 5; Fig. 1d). Only two AjEO–ciprofloxacin combinations and a combination of thymol and ciprofloxacin demonstrated synergy against *P. aeruginosa* ATCC 27853 (FICI = 0·37; FICI = 0·46), reducing MIC of ciprofloxacin by four to eightfold ($0{\cdot}125 \times 10^{-3}$, $0{\cdot}25 \times 10^{-3}$ *vs* 1×10^{-3} mg ml^{-1}) (Table 5).

Combination therapy in which antibiotics are associated with bacterial membrane permeabilizers, which enhance the antibiotics' penetration into the bacterial cell, seems to be very promising for the management of multidrug resistant bacterial infections (Hu *et al.* 2015). In this study, synergistic combinations of AjEO or thymol and amoxicillin against Gram-positive bacteria, such as *Staph. aureus* ATCC 25923 and MRSA 37, have been identified. A literature survey revealed few studies on the interactions between plant extracts and Amx against MRSA. Lemon grass essential oil, ethanolic extracts of *Emblica officinalis* seeds and *Nympha odorata* stamens showed synergistic activity with Amx against MRSA (Mandal *et al.* 2010; Abd El-Kalek and Mohamed 2012). The synergistic effects might be due to the fact that main constituents of AjEO and Amx target the bacterial cell wall: thymol, γ-terpinene and *p*-cymene damage the bacterial membrane by different mechanisms as previously described (Oyedemi *et al.* 2009; Hyldgaard *et al.* 2012) while Amx inhibits the bacterial cell wall synthesis (Clark *et al.* 2011). As our study revealed, the combination of AjEO with ciprofloxacin produces synergistic interactions against both Gram-negative (*P. aeruginosa* ATCC 27853) and Gram-positive bacteria (*Staph. aureus* ATCC 25923, *Strep. pneumoniae* clinical isolate). It is worthy to note the synergistic interaction against *P. aeruginosa*, a common agent for a broad spectrum of severe opportunistic infections, known for its resistance to many antibiotics. Similarly, synergistic effects have been reported for some carvacrol-rich essential oils (*Thymus maroccanus*, *Thymus broussonetii*) in combination with ciprofloxacin against *P. aeruginosa* clinical isolates (Fadli *et al.* 2012). With regard to *Staph. aureus*, few studies have described synergistic interactions for essential oils of *Lavandula angustifolia*, *Pelargonium graveolens*, *Thymus vulgaris* and *Mentha piperita* in combination with ciprofloxacin, dependent of combination ratios (van Vuuren *et al.* 2009; Malik *et al.* 2011; de Rapper *et al.* 2013). Ciprofloxacin, a broad-spectrum fluoroquinolone, acts by inhibition of DNA gyrase and topoisomerase IV, enzymes required for DNA replication, and also for the processes of transcription, repair and recombination. Phenolic compounds like thymol are able to alter not only the membrane and the cell walls, but also interfere with other bacterial cellular targets: protein and nucleic acid synthesis, enzyme activity, nutrient uptake and energy-generating processes (Souren *et al.* 2011). It is likely that synergistic antimicrobial interactions between AjEO and ciprofloxacin are caused not only by the membranotropic properties of the oil, but also by other mechanisms such as capacity to interfere with the bacterial metabolic pathways and enzymes or to enable a better diffusion of antibiotics through the bacterial membrane to reach specific cellular targets (Fadli *et al.* 2012). Although thymol is the main compound of AjEO and it is primarily responsible for its effects, the minor constituents can also significantly modulate the antibacterial activity. The profile of essential oil–antibiotic combination plays also a crucial role in the design of interaction type (van Vuuren *et al.* 2009).

Our study reveals for the first time the antibacterial effects of combinations based on AjEO/thymol and conventional antibiotics against multidrug resistant respiratory

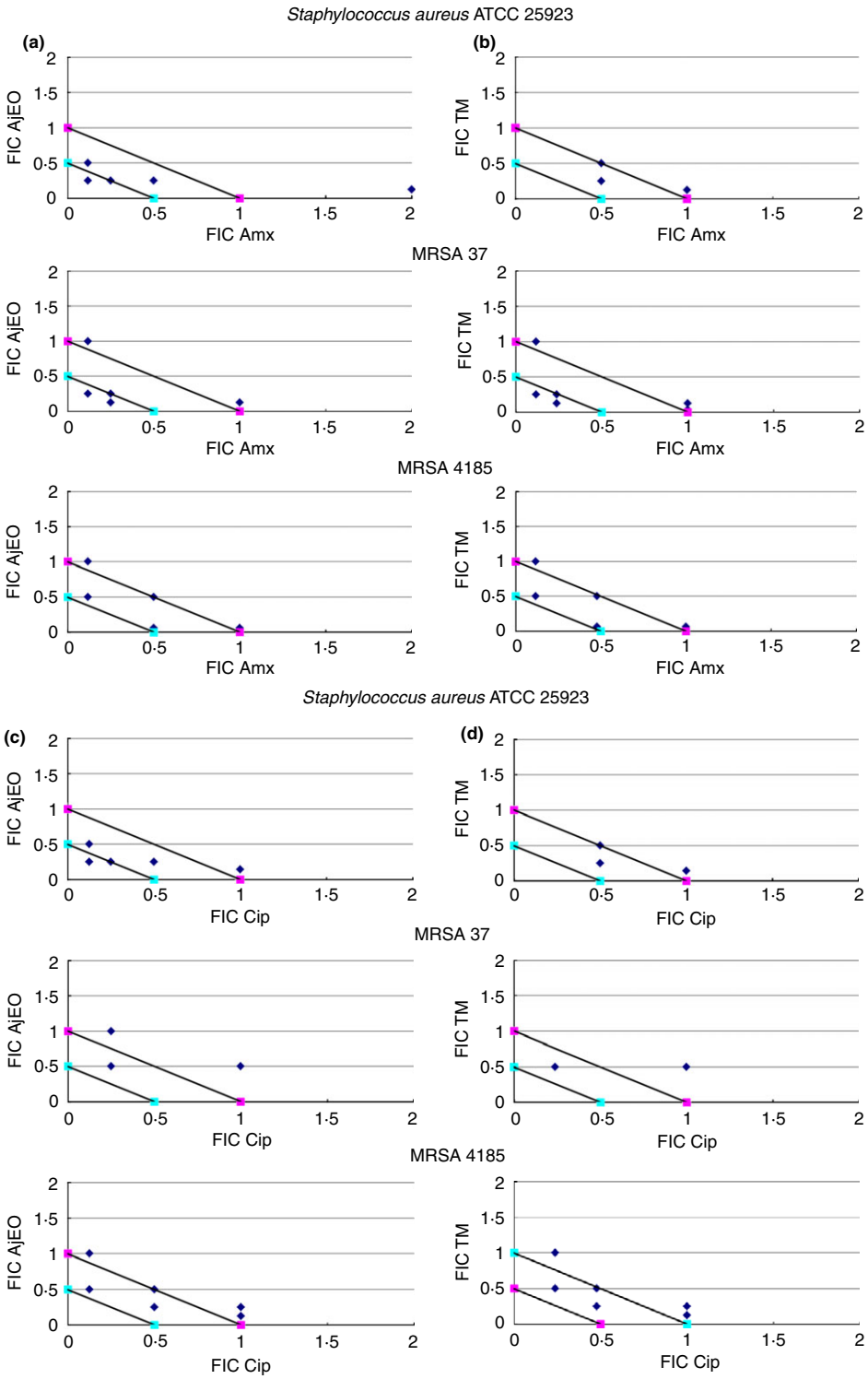

Figure 1 Isobolographic plot of (a) AjEO–Amx, (b) TM–Amx, (c) AjEO–Cip and (d) TM–Cip combinations against *Staphylococcus aureus* strains. FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index; AjEO, ajowan essential oil; TM, thymol; Cip, ciprofloxacin; Amx, amoxicillin. FICI points which have as coordinates FIC of AjEO and FIC of antibiotics or FIC of TM and FIC of antibiotics (blue diamonds); 0.5 values of FIC for both axes which define the synergy line (cyan squares); 1 values of FIC for both axes which define the addition line (magenta squares). Blue diamonds falling below or on 0.5:0.5 line correspond to synergistic interactions, those situated between 0.5:0.5 and 1:1 lines (including the ones on 1:1 line) to additive interactions, and those situated over 1:1 lines to other interactions (Schelz *et al.* 2006). [Colour figure can be viewed at wileyonlinelibrary.com]

Table 4 Effects of combinations between ajowan essential oil (AjEO)/thymol (TM) and amoxicillin (Amx)

Bacterial strains	Combination	FIC[1]/FIC[2]	FICI	Outcome
Staph. aureus ATCC 25923	AjEO[1]+Amx[2]	0·125/0·5	0·62	Ad
		0·125/0·25	0·37	S
	TM[1]+Amx[2]	0·5/0·25	0·75	Ad
MRSA 37	AjEO[1]+Amx[2]	0·12/0·25	0·37	S
		0·24/0·12	0·36	S
MRSA 4185	AjEO[1]+Amx[2]	0·5/0·06	0·56	Ad
	TM[1]+Amx[2]	0·48/0·06	0·54	Ad
PRSP 4423	AjEO[1]+Amx[2]	0·48/0·5	0·98	Ad
	TM[1]+Amx[2]	0·24/0·5	0·74	Ad

FIC, fractional inhibitory concentration; FICI, the fractional inhibitory concentration index; Ad, additive; S, synergistic; MRSA, methicillin-resistant *Staph. aureus;* PRSP, penicillin-resistant *Strep. pneumoniae.*

Table 5 Effects of combinations between ajowan essential oil (AjEO)/thymol (TM) and ciprofloxacin (Cip)

Bacterial strains	Combination	FIC[1]/FIC[2]	FICI	Outcome
P. aeruginosa ATCC 27853	AjEO[1]+Cip[2]	0·25/0·125	0·37	S
		0·5/0·125	0·62	Ad
	TM[1]+Cip[2]	0·21/0·25	0·46	S
		0·42/0·25	0·67	Ad
Staph. aureus ATCC 25923	AjEO[1]+Cip[2]	0·125/0·5	0·62	Ad
		0·125/0·25	0·37	S
	TM[1]+Cip[2]	0·5/0·25	0·75	Ad
MRSA 37	AjEO[1]+Cip[2]	0·25/0·5	0·75	Ad
	TM[1]+Cip[2]	0·24/0·5	0·74	Ad
MRSA 4185	AjEO[1]+Cip[2]	0·125/0·5	0·625	Ad
	TM[1]+Cip[2]	0·48/0·25	0·73	Ad
Strep. pneumoniae ATCC 49619	AjEO[1]+Cip[2]	0·24/0·5	0·74	Ad
	TM[1]+Cip[2]	0·25/0·5	0·75	Ad
PRSP 4423	AjEO[1]+Cip[2]	0·24/0·25	0·49	S
	TM[1]+Cip[2]	0·24/0·25	0·49	S
PRSP 4546	AjEO[1]+Cip[2]	0·25/0·5	0·75	Ad
		0·5/0·25	0·75	Ad
	TM[1]+Cip[2]	0·5/0·5	1	Ad
PRSP 4566	AjEO[1]+Cip[2]	0·24/0·5	0·74	Ad
	TM[1]+Cip[2]	0·24/0·5	0·74	Ad

FIC, fractional inhibitory concentration; FICI, the fractional inhibitory concentration index; Ad, additive; S, synergistic; MRSA, methicillin-resistant *Staph. aureus;* PRSP, penicillin-resistant *Strep. pneumoniae.*

pathogens. The results are encouraging because of the following findings: (i) AjEO and thymol are able to improve the effectiveness of ciprofloxacin in the treatment of infections with *P. aeruginosa*, *Staph. aureus* and P-resistant *Strep. pneumoniae* pathogens; (ii) AjEO and thymol can improve efficiency of Amx in the therapy of infections with *Staph. aureus* and Met-resistant *Staph. aureus* bacteria. The assessment of the therapeutic application of ajowan essential/thymol-antibiotics synergistic combinations potential requires further investigation to explore their functionality *in vivo* and to understand the synergy mechanism.

Materials and methods

Plant material

Dried ajowan fruits of Indian origin were purchased from a local supermarket and their botanical identity was confirmed in the Department of Pharmacognosy, Faculty of Pharmacy, *Grigore T. Popa* University of Medicine and Pharmacy Iasi, Romania. A voucher specimen (AJ no. 12013) was deposited in the same Department.

Essential oil extraction

The powdered dried fruits (100 g) were subjected to hydrodistillation for 3 h in a Clevenger-type apparatus. AjEO was dried over anhydrous sodium sulphate and stored in sealed glass tubes at 4°C until analysis. The essential oil yield was 7·4% (v/w).

Essential oil analysis

GC–MS analysis of AjEO was performed using an Agilent 7890A gas chromatograph, equipped with an Agilent 5975C mass spectrometer and a DB-5MS capillary column (30 m × 0·25 mm internal diameter, 0·25 μm film thickness). The carrier gas was helium with a flow rate of 1·5 ml min^{-1}. The injector and detector temperatures were maintained at 250°C. The oven temperature was raised, at a rate of 3°C min^{-1}, from 40 to 250°C (isothermal for 4 min) and then to 280°C at 10°C min^{-1}; the final temperature was hold for 2 min. GC analysis was carried out on an Agilent 6890 gas chromatograph equipped with a flame ionization detector (FID) in the same conditions as the ones used in GC–MS analysis. The essential oil constituents were identified by comparison of their mass spectra with those from the Wiley 275 Mass Spectral Library and of their retention indices relative to *n*-alkanes (C_8–C_{20}) with those mentioned in literature (Adams 2007; Goodner 2008).

Bacterial strains and antimicrobial susceptibility testing

The standard bacterial strains used in the study were: *P. aeruginosa* ATCC 27853, *Staph. aureus* ATCC 25923 and *Strep. pneumoniae* ATCC 49619 (Liofilchem, Abruzzi, Italy). The clinical isolates used were: *P. aeruginosa* 2351 (ARPA 2351), two strains of *Staph. aureus* (MRSA 37, MRSA 4185), and three of *Strep. pneumoniae* (PRSP 4423, PRSP 4546, PRSP 4566). They were obtained from pathological products (sputum, tracheobronchial aspirate) of patients with lower respiratory tract infections, and were

provided by the Microbiology Laboratory of the Lung Hospital, Iasi, Romania.

The antimicrobial susceptibility of clinical isolates was determined using the disc diffusion method on Mueller–Hinton agar (Oxoid, UK) and a panel of nine antibiotics: Amx (25 μg per disk), cefpirome (Cef; 30 μg per disk), doxycycline (Dox; 30 μg per disk), erythromycin (Ery; 15 μg per disk), levofloxacin (Lev; 5 μg per disk), Met (5 μg per disk), P (10 U per disk), streptomycin (St; 10 μg/disk) and vancomycin (Van; 30 μg per disk) (Oxoid). After the incubation for 16–18 or 24 h (*Strep. pneumoniae*) at 35°C, the growth inhibition zones were recorded and the results were interpreted according to Clinical and Laboratory Standards Institute guidelines (CLSI 2009).

Determination of the minimum inhibitory concentration

The MIC values were assessed by the broth microdilution method using 96-well plates (Deltalab, Barcelona, Spain) (Clinical and Laboratory Standards Institute (CLSI) 2009). Serial double dilutions of AjEO and thymol (Sigma-Aldrich, Steinheim, Germany), ranging from 64 to 0·0015 mg ml^{-1}, were prepared into Mueller–Hinton broth (Biolab Zrt., Budapest, Hungary), followed by inoculation (10^5 CFU per well). The solubility of AjEO was enhanced by adding 5% (v/v) DMSO to the 128 mg ml^{-1} stock solution of volatile oil in Mueller–Hinton broth. In each well, was added the inoculum adjusted to 0·5 McFarland standard turbidity. For *Strep. pneumoniae* isolates, the Mueller–Hinton broth was supplemented with 5% (v/v) lysed horse blood (Oxoid). Amoxicillin and ciprofloxacin (Bioanalyse Tibbi Malzemeler, Turkey) were used as positive controls in a dilution range from 64 to 0·007 μg ml^{-1}. The solvents used to dilute the antibiotics were phosphate buffer (pH = 6) for Amx and sterile double distilled water for ciprofloxacin. Negative controls were also included in the test, namely: bacteria growth control and sterility control, as well as solvents used to dilute samples and antibiotics. The final volume in each well was of 1 ml. The incubation was performed at 35°C, for 24 h. The amount of bacterial growth was monitored by visual assessment of turbidity. The MIC was defined as the lowest concentration of sample that inhibited the growth of the tested bacteria (Mighri *et al.* 2010). Each experiment was run in triplicate.

Checkerboard assay

This assay was performed to evaluate potential interactions between AjEO/thymol and antibiotics (Gradinaru *et al.* 2014). Serial double dilutions of AjEO, thymol and antibiotics were prepared as described previously for determination of MIC values. AjEO/thymol and antibiotics were dispensed on the 96-well plates in a checkerboard manner. Thus, AjEO/thymol was added to a Mueller–Hinton medium to give twofold dilutions along the *X*-axis, while the antibiotic was diluted twofold along the *Y* axis. The range of dilutions of AjEO, thymol and antibiotics comprises for each them the characteristic MIC value. Fifty microlitres of each dilution is found in each well, the final volume being of 100 μl. The inoculum adjusted to 0·5 McFarland standard of bacterial concentration was added to each well of the plate. The plates were incubated at 37°C for 24 h. The interactions between AjEO/thymol and antibiotics were interpreted on the basis of the fractional inhibitory concentration (FIC) index (FICI) values calculated as follows:

$FICI = FIC_{AjEO/thymol} + FIC_{antibiotic}$ where $FIC_{AjEO/thymol} = MIC_{AjEO/thymol\ in\ combination}/MIC_{AjEO/thymol\ alone}$ and $FIC_{antibiotic} = MIC_{antibiotic\ in\ combination}/MIC_{antibiotic\ alone}$. A combination of two substances operates synergistically if FICI $\leq$0·5, additively if 0·5 > FICI$\leq$1, indifferently if 1 > FICI$\leq$ 4 and antagonistically if FICI>4 (Schelz *et al.* 2006). The synergistic and additive interactions were illustrated using isobolograms. Points falling below or on 0·5 : 0·5 line correspond to synergistic interactions, those situated between 0·5 : 0·5 and 1 : 1 lines (including the ones on 1 : 1 line) to additive interactions.

Conflict of Interest

The authors declare no conflict of interest.

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